(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 December 2002 (19.12.2002)

PCT

(10) International Publication Number
WO 02/101387 A2

(51) International Patent Classification⁷: G01N 33/50

(21) International Application Number: PCT/IT02/00377

(22) International Filing Date: 7 June 2002 (07.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
RM01A000322 8 June 2001 (08.06.2001) IT

(71) Applicants *(for all designated States except US)*: CONSIGLIO NAZIONALE DELLE RICERCHE [IT/IT]; Piazzale Aldo Moro, 7, I-00185 Roma (IT). FERTILIA S.R.L. [IT/IT]; Via Paolo Gaifami, 10, I-95126 Catania (IT). LABOGEN S.A.S. DI CASTELLANO S.E. E C. [IT/IT]; Via Dottor Consoli, 68, I-95126 Catania (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: PARANO, Enrico [IT/IT]; Istituto Biolmmagini e Fisiopatologia del Sistema, Nervoso, Piazza Roma, 2, I-95123 Catania (IT). FALCIDIA, Ernesto [IT/IT]; Fertilia S.r.l., Via Paolo Gaifami, 10, I-95126 Catania (IT). GRILLO, Agata [IT/IT]; Labogen s.a.s di Castellano S.ll. & C., Via Dottor Consoli, 68, I-95124 Catania (IT).

(74) Agents: CAPASSO, Olga et al.; Ing. Barzano' & Zanardo Roma S.p.A., Via Piemonte, 26, I-00187 Roma (IT).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A NON INVASIVE METHOD FOR SCREENING AND DIAGNOSIS FETAL ANEUPLOIDIES

(57) Abstract: A non invasive method for screening and prenatal diagnosis 5 fetal aneuploidies including the following steps is described: collecting a maternal peripheral blood sample; separating fetal from maternal cells; identifying separated fetal cells; counting of identified fetal cells; assessing a sample exhibiting a number of fetal cells at least double than control one as positive with respect to foetal aneuploidy.

WO 02/101387 A2

A NON INVASIVE METHOD FOR SCREENING AND DIAGNOSIS OF FETAL ANEUPLOIDIES

The present invention relates to a non invasive method for screening and diagnosis of fetal aneuploidies. More particularly the invention relates to a method for prenatal screening and diagnosis performed on fetal cells from maternal blood samples, collected starting from 7th gestation week. The method proves to be quick, reliable, non invasive and suitable to be advantageously used for prenatal screening and diagnosis of fetal aneuploidies.

The invention is based on the finding that, when the fetus is affected by an aneuploidy, the number of fetal cells remarkably increase in maternal blood in comparison to normal physiological conditions. The authors found indeed that in pathological pregnancies a statistically and remarkably increased number of fetal cells in maternal blood is observed.

The authors of the present invention provide a method which allow to rapidly and reliably recognise and count fetal cells; this method represents a specific and reliable marker of fetal aneuploidy. The method allows fetuses suffering from chromosomal diseases to be easily identified and therefore to select pregnant women to be subjected to invasive tests, as amniocentesis or villocentesis. In fact about 70% of neonates affected by Down Syndrome (trisomy 21), which represents the human aneuploidy most frequently associated to mental retardation, are born from women less than 35 year old, which usually have no indication for invasive prenatal diagnosis. Most of these pregnant women indeed are addressed to non invasive prenatal screening (Triple-Test, nuchal Translucency, Ultrascreen), this tests, however, beside being not specific, present low reliability since may miss 15-40% of cases of Down Syndrome in addition to having a 5-8% rate of false-positives results [1-7, 14-15].

It has been showed and confirmed that fetal cells, like Fetal Nucleated Erythrocytes (FNRBCs), Leucocytes and Trophoblasts are present in the maternal blood during the pregnancy [1-5; 8-13]. We have reported that the number of cells and/or the amount of fetal DNA is

remarkably increased in maternal blood when the fetus is affected by aneuploidy, especially Trisomy 21, 13 and 18 [14-15] (publications after patent). However, before present, these data were not statistically significant and do not showed any technical features resulting in a simple, rapid, reliable and reproducible method suitable for identifying and counting fetal cells in order to carry out a non invasive prenatal screening to identify fetuses affected by aneuploidies.

The authors of the present invention provide a screening test suitable to be used alone or in association with other currently used serological screening methods.

It is therefore an object of the instant invention a non invasive method for prenatal screening and diagnosis of fetal aneuploidies, including the following steps:

a) collecting a maternal peripheral blood sample;

b) separating fetal from maternal cells from said sample;

c) identifying separated fetal cells;

d) counting identified fetal cells;

e) assessing as positive with respect to fetal aneuploidy samples containing a number of fetal cells at least double than control.

Separation methods advantageously usable comprise MACS (Magnetic Activated cell sorting method); FACS (Fluorescence Activated Cell Sorting Method); density based separation methods using continuous or non continuous gradients (for example Ficoll, Percoll, Hispopaque, Lymphoprep). Those expert in the field will also appreciate that other separation methods are within the scope of the present invention.

Identification methods advantageously usable comprise histochemical methods (for example staining for fetal haemoglobin, HbF), and May Grumwald Giemsa staining methods, which allow fetal erythrocytes to be identified respectively on the basis of colorimetric staining *(HbF)* and morphological characteristics (May Grumwald Giemsa); immunohistochemical methods (for example using fluorescent markers allowing various cell populations to be identified and distinguished or specific structures within the cells to be identified). Those expert in the

field will appreciate that other identification methods are within the scope of the present invention.

Methods allowing fetal cells to be counted simply, reliably and reproducibly are those using optical microscopy apparatuses (using inverted or transmitted light, manual or automated); optical fluorescence (manual or automated); scanning and transmission electron microscopes. Those expert in the field will appreciate that fetal cell counting is within the scope of the present invention. Suitable kit to perform the disclosed are also comprised within the scope of the invention.

The present invention will be now described by way of illustrative but not limitative examples with reference to figure 1, wherein panel A shows a FNRBC selected cell after HbF staining (100x); panel B shows a FNRBC selected cell after May-Grunwald-Giemsa staining.

Materials and Methods

(Group I). Blood samples from 50 pregnant women, subjected to amniocentesis or villocentesis, selected on the basis of high fetal aneuploidy risk (one or more of the following reasons: age over 40 years and/or positive fetal ultrasound scanning, Triple Test, Ultrascreen, NT). For any sample previous informed consensus was achieved.

(Group II). Blood samples defined "cases" were also similarly collected from 30 women with a diagnosed aneuploidy by invasive methods (villocentesis/amniocentesis), with parallel 30 blood samples defined "controls" from women having comparable age, pregnancy age, and type of invasive diagnosis performed.

All blood samples (8 ml) were collected immediately after results of invasive diagnosis and subjected to the following procedure: samples were kept at ambient temperature for 5 hours or at 4°C for one night. In order to eliminate most of non fetal red line cells (non-FNRBCs) blood aliquots were layered on double density non continuous Ficoll gradient (1.077 g/ml and 1.083 g/ml) (Sigma Diagnostic, St. Louis, MO; USA) and centrifuged. Cells at 1.077 and 1.083 g/ml interface gradients were collected using Pasteur pipette (about 2 ml) and PBS twice washed. Two blood smears were put on slide and microscope inspected. To

identify FNRBCs cells any slide was HbF stained (minor modification of the Fetal Cell Stain Kit, Simmler, Inc., St. Louis, MO, USA). FNRBCs cells were identified based on a deep red staining, unlike other pink or non stained cells (Fig. 1A). FNRBCs were then marked and the slides washed and May-Grunwald-Giemsa re-stained allowing to confirm the FNRBCs position by morphological characteristics thereof (Fig. 1B). Recognised FNRBCs then were finally counted.

Results

Group I: Results show that the number FNRBCs cells contained in the blood samples from 50 selected pregnant women presents a remarkable bimodal distribution: for 43 cases 40 FNRBCs on average were counted (range 30-50); on the contrary for 7 cases the average was higher: 242 FNRBCs (range 212-272). Amniocentesis results from 50 selected pregnant women showed 43 normal karyotypes, corresponding to 43 examined samples wherein FNRBCs average number was 40. As for 7 remaining cases amniocentesis showed 7 aneuploidy cases: namely, three Trisomy 21 (one Ultrascreen and two Triple-Test already positive women) two Klinefelter Syndrome (a 42 year old and a pregnant woman for which the fetal ultrasound scanning had shown cerebral fetal anomalies); and one Trisomy 13 (a pregnant woman for which the fetal ultrasound scanning had shown cardiac anomalies); one Trisomy 18 cases (a Ultrascreen positive pregnant woman). All these 7 cases were corresponding to the blood samples with high FNRBCs number (over 5 times than normal).

Group II: Results of Fetal Cells Count show that the number of FNRBCs present in the blood samples analyzed has shown a bimodal distribution, with marked increase number of FNRBCs (from 260 to over 300 FNRBCs) within 30 cases corresponding to mothers in which invasive prenatal diagnosis (amniocentesis and/or villocentesis) had shown a fetal aneuploidy (22 cases of Down syndrome; 3 cases of Klinefelter syndrome; 2 cases of trisomy 13; 2 cases of trisomy 18; 1 case of Turner syndrome) , while a much less number of FNRBCs was present within the corresponding 30 controls (average 36).

Results obtained show that in cases of aneuploidic fetuses, including Trisomy 18, Trisomy 13, Klinefelter Syndrome, Turner Syndrome and especially Trisomy 21, a great increase number of FNRBCs is already evident at 13-14 weeks of gestation. Taken together with preliminary reported and published results, these data confirm the evidence that the number of FNRBCs is markedly increase in blood samples of mother bearing an aneuploidic fetus (approximately 6-time normal and even more). Then it has been demonstrated that FCC might be considered a reliable and effective screening test of aneuploidy. This test alone or in concert with results of other maternal screening tests (Triple test, Ultrascreen) would improve detection rate of aneuploidies.

Diagnostic kit

A kit to perform the method as disclosed above could comprise:

1) syringe;
2) test tube to centrifuge and perform the ficoll sparation;
3) Automatic pipet dispenser to isolate the FNRBC enriched zone
4) Glass slides and dyes (HBF e May Gru.) to identify FNRBCs

Advantageously microscopes could be adapted to automatically count FNRBCs in relation to colorimetric and morphological features thereof.

BIBLIOGRAPHY

1. Al-Mufti R., Hambley H, Farzaneh F., Nicolaides K. Investigation of maternal blood enriched for fetal cells: role in screening and diagnosis of fetal trisomies. Am J Med Genet 1999; 85: 66-75.

2. Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW, Shuber AP. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. Am J Hum Genet 1997; 61: 822-829.

3. Bianchi DW. Fetal cells in the maternal circulation: feasibility for prenatal diagnosis. BrJ Haematol 1999:105: 574-583.

4. Farina A, Bianchi DW. Fetal cells in maternal blood as a second non-invasive step for fetal Down syndrome screening. Prenat Diagn 1998; 18 :983-984.

5. Oosterwijk JC, Masker WE, Ouwerkerk-van Velzen MCM, Knepfle CF, Wiesmeijer KC, Beverstock GC, van Ommen GJB, Tanke HJ. Kanhai HH. Prenatal diagnosis of trisomy 13 on fetal cells obtained from maternal blood after minor enrichment. Prenat Diagn 1998; 18: 1082-1085.

6. Roberts L, Sebire NJ, Fowler D, Nicolaides KH. Histomorphological features of chorionic villi at 10-14 weeks of gestation in trisomic and chromosomally normal pregnancies. Placenta 2000; 21: 678-683.

7. Simpson JL, & Etias S Prenatal diagnosis of aneuploidy using fetal cells isolated from maternal blood. University of Tennessee, Memphis experience. Ann N Y Acad Sci 1994; 731: 80-91

8. Simpson JL, & Etias S. Isolating fetal cells from maternal blood. Advances in prenatal diagnosis through molecular technology. JAMA 1993:270:2357-2361.

9. Takabayashi H, Kuwabara S, Ukita T, Kazumi K, Yamafuji K, Igarashi T.. Development of non-invasive fetal DNA diagnosis from maternal blood. Prenat Diagn 1995; 15: 74-77

10. Zhong XY, Burk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. Prenat Diagn. 2000; 20:795-8

11. Lagena F, Smid M, De Benassuti L, Ferrari A, Ferrari M, Cremonesi L. Fetal DNA analysis from maternal plasma and nucleated blood cells. Ann N Y Acad Sci. 2000 Apr; 906:156-60.

12. Lo YM. Fetal DNA in maternal plasma. Ann N Y Acad Sci. 2000 Apr; 906:141-7

13. Lo YM. Fetal DNA in maternal plasma: biology and diagnostic applications. Clin Chem. 2000; 46(12): 1903-6.

14. Parano E, Falcidia E, Grillo A, Takabayashi H, Trifiletti RR, Pavone P. Fetal nucleated red blood cell counts in peripheral blood of

mothers bearing Down syndrome fetus. Neuropediatrics. 2001 32(3):147-9.

15. Parano E, Falcidia E, Grillo A, Pavone P, Cutuli N, Takabayashi H, Trifiletti RR, Gilliam CT. Noninvasive prenatal diagnosis of chromosomal aneuploidies by isolation and analysis of fetal cells from maternal blood. Am J Med Genet. 2001 1;101(3):262-7.

CLAIMS

1. A non invasive method for prenatal screening and diagnosing fetal aneuploidies including the following steps of:

a) collecting a maternal peripheral blood sample;

b) separating fetal from maternal cells from said sample;

c) identifying separated fetal cells;

d) counting identified fetal cells;

e) assessing as positive with respect to fetal aneuploidy samples containing a number of fetal cells at least double than control.

2. Method for prenatal diagnosing foetal aneuploidies according to claim 1 wherein the separation step includes MACS method (Magnetic Activated cell sorting method); FACS method (Fluorescence Activated Cell Sorting Method); or density based separation methods using continuous or non continuous gradients (for example Ficoll, Percoll, Hispopaque, Lymphoprep).

3. Method for prenatal diagnosis fetal aneuploidies according to claim 2 wherein the separation step is carried out based on density using non continuous gradient.

4. Method for prenatal diagnosis fetal aneuploidies according to claim 1 wherein the fetal cell identification step includes histochemical methods (for example fetal haemoglobin (HbF) and May Grumwald Giemsa staining methods); immunohistochemical and fluorescence methods (for example by means of fluorescent markers allowing various cell populations to be identified and distinguished or specific structures within the cells to be identified).

5. Method for prenatal diagnosis fetal aneuploidies according to claim 4 wherein the foetal cell identification step includes histochemical fetal haemoglobin staining methods.

6. Method for prenatal diagnosis fetal aneuploidies according to claim 1 wherein the foetal identified cell counting step is carried out by means of microscopy apparatuses as inverted or transmitted light optical, manual or automated; fluorescence optical, manual or automated; or scanning and transmission electron microscopes.

7. Method for prenatal diagnosis fetal aneuploidies according to claim 6 wherein the fetal cell counting step is carried out by means of optical microscopy.

8. A kit to perform the method acoording to any of previous claims comprising:

1) syringe;
2) test tube to centrifuge and perform the ficoll sparation;
3) automatic pipet dispenser to isolate the FNRBC enriched zone;
4) glass slides and dyes (HBF e May Gru.) to identify FNRBCs.

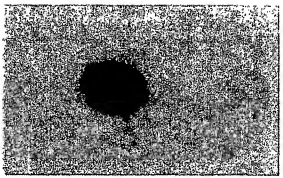

Fig.1a

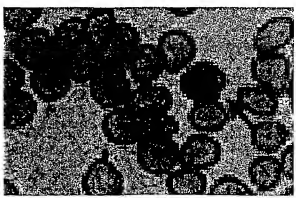

Fig.1b